New PCT Patent Application
CropDesign N.V.
Our Ref.: D 1321 PCT

Method for enhancing and/or improving plant growth and/or yield or modifying plant architecture

Field of the invention

The current invention relates to a method for modifying, preferably for enhancing or promoting plant growth and/or yield in plants and for modifying their architecture and to the transgenic plants obtainable by this method.

The invention concerns the simultaneous ectopic expression and/or overexpression of at least two cell cycle interacting proteins capable of forming a complex and specifically a CDK and an interacting cyclin; said co-overexpression results in an unexpected growth and architectural characteristics such as enhanced root and/or shoot growth in plants.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

Background of the invention

Cell cycle – General

Higher plants are multicellular organisms, hence their growth is a function of the rate at which new cells are produced. There is no doubt that the regulation of the cell division cycle plays a crucial role in plant growth regulation.

The eukaryotic cell division cycle can be divided in four main phases: the G1 phase or first gap phase, the S phase during which the DNA is duplicated, the G2 phase or second gap phase, and the M phase during which karyo- and cytokinesis take place. The major checkpoints regulating the progression through the cell cycle are situated at the G1/S and G2/M transitions. If the conditions are inadequate for the cell to continue its cycle, a block can occur at one or both transition points. The cell cycle can also be blocked at other transition points which had not until recently been considered important, namely M/G1. Passage through the G1/S and G2/M boundaries is dependent on the activity of cyclin dependent kinases (CDKs). A prerequisite for CDK activity, which by itself is regulated by phosphorylation and dephosphorylation events, is binding to a regulatory subunit, a cyclin. Generally cyclins oscillate at both transcriptional and protein level in a cell cycle phase dependent manner. The association of a cyclin with a CDK not only confers activity but also contributes to the substrate specificity of the CDK complex and its subcellular localisation.

CDKs

Intensive cloning efforts have identified a large number of CDK proteins in diverse plant species, among which at least five types can be distinguished on the basis of their sequences (for a compilation see Segers et al., 1997). In the model plant, *Arabidopsis thaliana,* two CDKs, each belonging to a different family, have been characterised. One such example is the *CDC2a*At gene, which contains the conserved PSTAIRE amino acid motif, and is constitutively expressed during the cell cycle at transcriptional and protein level. However, the associated kinase activity is maximal at the G1/S and G2/M transitions, suggesting a role at both checkpoints (Hemerly *et al.*, 1993; Burssens et al., 1998; Segers *et al.*; 1996). *CDC2b*At contains a PPTALRE motif and its mRNA levels are preferentially present during S and G2 phase (Segers *et al.*, 1996 and references cited therein). The protein follows the transcriptional level but the CDC2b*At* kinase activity becomes only maximal during mitosis, implying a role during the M phase.

Cyclins

Numerous cDNAs encoding putative cyclins have been found in a diverse range of plant species, amounting up to 100 with at least 15 representatives in *Arabidopsis* alone (for a compilation, see Renaudin et al., 1996). Cyclins share a highly homologous region of about 100 amino acids termed the "cyclin box" which is required for their interaction with the CDK catalytic subunit. Analysis of the deduced peptide sequences in the conserved cyclin box has enabled the classification of these cyclins into nine groups: A1, A2, A3, B1, B2, D1, D2, D3, and D4 reflecting their homologies to the mammalian cyclins A, B, and D (Renaudin et al., 1996; Burssens et al., 1998; De Veylder et al., 1999 and references cited therein). See also Table 1 for a list of cyclins relevant for the invention. Cyclins with homology to mammalian Cyclin C have been recently identified in rice and *Arabidopsis,* thus adding even more complexity to the family of cyclins in plants.

The plant A- and B- type cyclins are the so called 'mitotic cyclins' with an important function during mitosis, while the D-type cyclins (so called G1 cyclins) are thought to play a key role at the entry of S phase. The transcriptional regulation of the mitotic cyclins *CYCA2;1* and *CYCB1;1* of *A. thaliana* has been analysed in detail in synchronised tobacco BY2-cells. Promoter activity of *CYCA2; 1* is switched on upon entry of S phase and persists during G2 phase to be maximal at the end of G2 phase. *CYCB1;1* is expressed in a more narrow window of the cell cycle, namely upon exit of S phase and G2 phase with maximal mRNA levels at the entry of mitosis (Shaul *et al* 1996). Moreover, by developmental expression analysis, the presence of *CYCB1;1* transcripts was exclusively linked with actively dividing cells (Ferreira *et al.*, 1994), implying that *CYCB1;1* is involved in the regulation of mitosis. Plant D cyclins, by analogy with their animal homologues, have been proposed to control the G1 progression in response to growth factors and nutrients (Dahl et al., 1995; Soni et al., 1995). Moreover ectopic expression has been shown to render cell division in *Arabidopsis* independent of the growth

hormone cytokinin (Riou-Khamlichi et al 1999). Cyclins CYCD2;1 and CYCD3;1 from tobacco are found to be expressed predominantly in G-M (Sorrell et al., 1999), suggesting that D-type cyclins in plants may also be involved in mitotic events. A novel cyclin CYCD4 from Arabidopsis has also been identified and is shown to be expressed during vascular tissue development, embryogenesis, and formation of lateral root primordia (De Veylder et al. 1999).

CDK/Cyclin complexes

There is evidence showing that CDKs and cyclins interact to form functional protein complexes. Bögre et al. (1997) have found that protein fractions from alfalfa extracts corresponding to monomeric CDKs are essentially devoid of kinase activity as measured by histone H1 phosphorylation and, on the other hand, alfalfa protein complexes immunoprecipitated with antibodies against the human cyclin A or alfalfa cyclin CYCB2;2 exhibit appropriate histone H1 kinase activity (Magyar et al., 1993; 1997). Immunolocalization of CDC2Zm and mitotic cyclins in maize suggest the occurrence of several possible combinations of CDKs and cyclins (Mews et al 1997). Further, the inventors have shown, using the two-hybrid system, the interaction of CYCD1;1 (De Veylder et al., 1997a) and CYCD4;1 (De Veylder et al., 1999) with CDC2aAt. Additionally, the inventors are able to purify active kinase complexes from Arabidopsis cells that contain selectively either CDC2aAt or CDC2bAt. The following protein complexes have been purified: CDC2a with CYCB2;2, CDC2a with CYCA2;2, CDC2b with CYCB1;1, CDC2b with CYCA2;2.

Other cell cycle complexes

A number of other cell cycle proteins have been shown to interact with one another to form active complexes. Some complexes relevant to the invention are involved in the initiation of DNA replication and cell division including facilitating the entry into S phase of quiessant cells (see Leatherwood 1998, Helin 1998 for reviews). The complexes include ORC1/CDC6 or CDC7/DBF4 or E2F/DP.

Importance of the cell cycle

The presence of multiple cyclins and CDKs enables the fine regulation of cell cycle controls and checkpoints since different kinase activities are involved at different points within the cell cycle (Burssens et al, 1998 and references cited therein). The importance of the cell cycle for plant growth and development is illustrated by the observed growth inhibition in response to chemical and radiation treatments, that specifically block cell cycle progression (Foard and Haber, 1961b; Ivanov, 1994). Moreover, as in yeast and animal systems (for an overview see e.g. (Murray and Hunt, 1993)), it is expected that the majority of mutations of cell cycle genes are either lethal or result in severe growth reductions. Inversely, if the cell cycle plays a role in plant growth regulation, it is possible to modify plant growth rates by manipulating the expression of cell cycle genes that are limiting cell division and thereby plant growth rates. Indeed, Doerner, 1996 has suggested that ectopic expression of *CYCB1;1* under the control of the *CDC2a* promoter in *A. thaliana* plants accelerates root growth without altering the pattern of lateral root development or inducing neoplasia. In contrast to this data, the inventors have shown that constitutive overexpression of *CYCB1;1* alone does not lead to any significant growth rate differences in at least two independently transformed lines.

CDK activity depends on the interaction with regulatory cyclins and is limiting for cell cycle progression. Different CDK/cyclin complexes act at different time-points of the cell cycle, although different CDK/cyclin complexes may also act at the same time-points of the cell cycle. Complexes of A-type CDKs (such as CDC2a) with D-type cyclins are acting at the G1 phase and are involved in recruiting G0 cells in the G1 phase of the cell cycle. Complexes between A-type CDKs and A-type cyclins are operational in S- and G2 phase. At G2- and M-phase complexes between A-and B-type CDKs and B-type cyclins are controlling the progression of the cell cycle.

Two major checkpoints are operational during the cell cycle, one at the G1/S boundary and one at the G2/M boundary. At these checkpoints the activity of the appropriate CDK/cyclin complexes is controlled, either by interaction with inhibitory proteins (at the G1/S transition), or by inhibitory phosphorylations mediated by a Wee1 kinase (at the G2/M transition). Only when the conditions are favourable is the CDK/cyclin kinase activity restored either by inactivation of the inhibitor, or by dephosphorylation of the CDK/cyclin complex through the action of CDC25.

The current invention describes methods to overcome the inhibition of CDK/cyclin activity. We disclose that the simultaneous (over)expression of a CDK and its regulatory cyclin overrides the potential inhibition of the CDK/cyclin complex at the checkpoints. Depending on the specific CDK/cyclin combination different effects are expected in plant growth, yield or architecture.

Thus, the technical problem underlying the present invention is to provide means and methods for enhancement of plant growth, and/or yield and/or modified architecture in particular in the entire plant, or specific parts of said plant, which are particularly useful in agriculture.

The solution to the technical problem is achieved by providing the embodiments characterised in the claims.

SUMMARY OF THE INVENTION

The present invention relates to a method for modifying plant growth and/or yield and/or architecture, in particular modifications to plant growth and development mediated by cell cycle protein complexes, thereby improving the agricultural and commercial value of these plants. Surprisingly, it has been found that the overexpression of at least two proteins forming subunits of a

protein complex in particular cells, tissues or organs of the plant would produce enhanced plant growth and/or yield and/or architecture compared to otherwise non-transformed plants.

Accordingly, the present invention relates to a method for modifying plant growth and/or yield and/or modifying architecture comprising introducing into a plant cell, plant tissue or plant (a) nucleic acid molecule(s) or regulatory sequence(s), wherein the introduction of the nucleic acid molecule(s) or regulatory sequence(s) result(s) in an increased or de novo expression of at least two cell cycle interacting proteins capable of forming a (heteromeric) complex in a plant cell.

Surprisingly, it has been found that the simultaneous ectopic expression and/or overexpression of more than one cell cycle interacting protein, of which, preferably, at least one is a protein kinase and at least one is a protein forming a complex with such protein kinase and regulating the activity of said protein kinase, leads to enhanced plant growth and/or yield and/or modified architecture compared to wild type plants.

This effect is surprising in the light of the fact that (i) CDC2a is known to be constitutively expressed throughout the cell cycle and therefore not obviously a rate limiting factor for cell division and (ii) Doerner et al. 1996 have shown growth stimulation based on ectopic overexpression of CYCB1;1 alone. The inventors have shown that, under their experimental conditions, no growth stimulating effect of CYCB1;1 was observed whereas a growth stimulation was observed with simultaneous (over)expression of both CYCB1;1 and CDC2a. Even when one assumes that CYCB1;1 overexpression may have a growth stimulating effect in itself under certain conditions, the current invention demonstrates that it will be advantageous to use co-expression of both CYCB1;1 and CDC2a since this leads to growth stimulation under conditions in which CYCB1;1 alone does not stimulate growth. The combined

overexpression may therefore stimulate growth under a wider range of conditions, including environmental conditions, such as high or low availability of water and nutrients, high or low temperature, high or low light, etc.

In a preferred embodiment of this invention, the protein kinase is a cyclin dependent kinase (CDK). In a more preferred embodiment, the CDK is a PSTAIRE type cyclin dependent kinase. In a most preferred embodiment the CDK is a CDC2a. In an alternatively preferred embodiment, the CDK is a B-type cyclin dependent kinase namely CDC2b.

In a further preferred embodiment of this invention the second protein is a cyclin. In a more preferred embodiment the cyclin is a G1 cyclin such as a D-type cyclin (e.g. CYCD4;1) or an E-type cyclin. In an alternatively preferred embodiment of the invention the cyclin is a mitotic cyclin such as a B-type cyclin like CYCB1;1.

In a further preferred embodiment the cell cycle interacting proteins to be coexpressed and forming the relevant cell cycle complex are ORC1 with CDC6 or CDC7 with DBF4 or E2F with DP.

In a further preferred embodiment of the invention both proteins of the cell cycle complex are ectopically expressed under the control of a constitutive promoter such as the 35S promoter. It will be clear to the man skilled in the art that both proteins may also be expressed under the control of other promoters such as tissue specific promoters, which may be the same for both proteins or which may be different as long as those promoters are driving simultaneous expression of both proteins in at least one tissue. Growth stimulation occurs in particular in those tissues in which both proteins are simultaneously (over)expressed.

The man skilled in the art will see various ways of implementing said method in plants.

In one embodiment of the invention plants are transformed separately with one protein of the cell cycle complex, e.g. or protein kinase such as CDC2a, and with the other interacting cell cycle protein of the complex such as a regulatory protein of such protein or protein kinase, e.g. CYCD4;1. Such plants are subsequently crossed and the offspring plants that contain both transgenes are selected and demonstrated to exhibit modified growth, yield and/or architectural characteristics in comparison with a wild type plant.

In another embodiment of the invention plants exhibiting simultaneous ectopic expression and/or overexpression of the two genes according to the present invention will be obtained via so called cotransformation. Each gene will be present in a different vector or gene construct (e.g. an *Agrobacterium* vector) and during the transformation step both vectors will be used in combination. The success rate of cotransformation will be highest when both vectors contain a different marker gene (e.g. bar, nptII, hyg,..) and when the selection will be performed with both selective agents; it is also possible to use only one selectable marker gene and its corresponding selective agent and then to identify cotransformants by means of genetic analysis (e.g. PCR based methods). Alternatively each gene will be present on the same vector or gene construct and the plant transformed with such a vector.

In yet another embodiment of the invention specific vectors will be constructed in accordance with the invention. Such vectors will contain a nucleic acid molecule, e.g. a gene, encoding one protein of the cell cycle complex, e.g. encoding a protein kinase, under the control of a given promoter sequence as well as a nucleic acid molecule, e.g. a gene, encoding the other interacting cell cycle protein of the said complex, e.g. regulating the protein kinase activity, under the control of a given promoter sequence. In

addition to the promoters other control sequences may be present. The promoters of both genes may be identical or may be different as long as there is simultaneous expression in at least one tissue. Bidirectional promoters such as the TR promoter may also be used to drive expression of both genes.

The transgenic plants, plant tissues, plant organs or plant cells obtained by the method according to the invention are obtainable from a monocotyledonous plant or dicotyledonous plant.

The invention also relates to a transgenic plant cell comprising an overexpressed protein complex obtainable according to any of the methods of the present invention. A transgenic plant or plant tissue comprising said plant cells and harvestable parts or propagation material of those plants are part of the invention too.

The invention also relates to the vectors necessary to obtain transformed plants in accordance with the previous embodiments of the invention, those vectors are characterized by the fact that they contain both a protein kinase gene and a gene encoding a regulatory protein regulating the activity of said protein kinase.

The invention is also related to utilisation in hybrid seeds in the following way. Two transgenes of interest, each present in a homozygous condition in one of the parents of a hybrid, will be present in combination and in a heterozygous condition in the hybrid seed, thus providing the hybrid seed with the benefit of accelerated growth based on the simultaneous ectopic expression and/or overexpression of the two transgenes. Seed harvested from the F1 hybrid plants will segregate for both transgenes and only 9 out of 16 plants of the F2 generation will possess the two transgenes, thus resulting in additional protection of the value of the hybrid seed.

In a still further embodiment the present invention relates to composition comprising the above-described nucleic acid molecules, regulatory sequences or vectors, containing the same identified by the method of the present invention.

In another embodiment the invention relates to the use of the transformed cells or the above described nucleic acid molecules, regulatory sequences or vectors for the production of more biomass, secondary metabolites or additives for plant culturing in plant cell culture.

Detailed description of the invention

The present invention relates to a method for modifying plant growth and/or yield and/or modifying architecture comprising introducing into a plant cell, plant tissue or plant (a) nucleic acid molecule(s) or regulatory sequence(s), wherein the introduction of the nucleic acid molecule(s) or regulatory sequence(s) result(s) in an increased or de novo expression of at least two cell cycle interacting proteins capable of forming a (heteromeric) complex in a plant cell.

The term "growth" is a concept well known to the person skilled in the art and includes increased crop growth and/or enhanced biomass.

The term "modifying plant growth and/or yield" refers to a general alteration in the growth of the plant, its tissues or organs or the yield as examplified below. Preferably, "modifying plant growth" relates to an acceleration, enhancement or promotion of plant growth.

"Architecture" refers to the general morphology of a plant including any one of more structural features including the shape, size, number, colour, texture, arrangement and patternation of any cell, tissue or organ or groups of cells,

tissues, or organs of plants including the root, leaf, shoot, fruit, petiole, trichome, flower, sepals, petal, hypocotyl, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre nodule, cambium, wood, heartwood, parenchyma, erenchyma, selve element, phloem, or vascular tissue amongst others.

"Modifying yield" refers to an altered, preferably increased or enhanced biomass of either the total plant or specific tissues or organs of plants such as root, leaf, shoot, fruit, petiole, trichome, flower, sepals, petal, hypocotyl, stigma, style, stamen, pollen, ovule, seed, bulb, embryo, endosperm, seed coat, aleurone, fibre, nodule, cambium, wood, heartwood, parenchyma, erenchyma, seive element, phloem, or vascular tissue. "Yield" also refers to accumulation of metabolites and/or the sink/source relationships in the total plant or specific cells, tissues or organs of the plant such as root, leaf, shoot, fruit, petiole, trichome, flower, sepals, petal, hypocotyl, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, nodule, cambium, wood, heartwood, parenchyma, erenchyma, seive element, phloem, or vascular tissue. This means, for instance that increased growth and/or yield results from increased growth rate or increased root size or shoot growth or, alternatively, in an increased yield because of enhanced fruit growth.

As will be known to those skilled in the art, plants may modify one or more plant growth and/or architectural and/or yield characteristics in response to external stimuli, such as, for example, a plant pathogenic infection, or an external stress or environmental stress (e.g. anoxia, hypoxia, high temperature, low temperatures, light, daylength, drought, flooding, salt stress, dehydration, heavy metal contamination, nutrient/mineral deficiency, amongst others). Accordingly, for the present purpose, it shall be understood that a plant growth or architectural or yield characteristic that has been modified in response to one or more external stimuli is within the scope of the inventive

method described herein, notwithstanding that the imposition of said external stimuli is not an essential feature of the present invention.

The term "simultaneous" as used in the context of "simultaneous ectopic expression and/or overexpression" or "simultaneous (over)expression" shall mean preferably at the same time and in the same cells but at least that, although not expressed at the same time, there is an overlap in the timing of the existence of the proteins of interest so that the proteins are capable of forming a complex.

The term "cell cycle" as used herein shall be taken to include the cyclic biochemical and structural events associated with growth and with division of cells, and in particular with the regulation of the replication of DNA and mitosis. Cell cycle includes phases called: G0 (gap 0), G1 (gap 1), DNA replication (S), G2 (gap 2), and mitosis including cytokinesis (M). Normally these four phases occur sequentially. However, the cell cycle also includes modified cycles such as endomitosis, acytokinesis, polyploidy, polyteny, endopolyploidisation and endoreduplication or endoreplication.

The term "cell cycle interacting protein", "cell cycle protein", or "cell cycle control protein" as denoted herein means a protein which exerts control on or regulates or is required for the cell cycle or part thereof, of a cell, tissue, organ or whole organism and/or DNA replication. It may also be capable of binding to, regulating or being regulated by cyclin dependent kinases or their subunits. The term also includes peptides, polypeptides, fragments, variants, homologues, alleles or precursors (e.g. preproproteins or preproteins) thereof. The cell cycle interacting protein is preferably of plant origin, although it may also be the yeast homologues thereof.

The cell cycle interacting protein is preferably a protein kinase, in particular a cyclin dependent kinase (CDK) for example an A-type (CDC2a) or a B-type (CDC2b) CDK.

The cell cycle interacting protein is also preferably a cyclin, including cyclins A, B, C, D and E, and in particular CYCA1;1, CYCA2;1, CYCA3;1, CYCB1;1, CYCB1;2, CYCB2;2, CYCD1;1, CYCD2;1. CYCD3;1, and CYCD4;1 (Evans *et al.* 1983; Francis *et al.* 1998; Labbe *et al.* 1989; Murray and Kirschner 1989; Renaudin *et al* 1996; Soni *et al* 1995; Sorrell *et al* 1999; Swenson *et al* 1986). Cyclins are also referred to in Table 1.

The cell cycle interacting protein preferably includes proteins involved in the control of entry and progression through S phase in particular ORC1, CDC6, (Nevins, 1992; Liang, 1995) CDC7, DBF4 kinase, E2F (WO 99/58681; WO 99/53075) and DP (WO 99/53075).

The term "capable of forming a (heteromeric) complex" as used herein means that said at least two cell cycle interacting proteins bind, interact or associate with each other to form a complex in a cellular, preferably plant cellular environment. Preferably, the complex formation in the cell leads to the induction of potential processes of cell division, preferably cell proliferation. Examples of such cell cycle interacting proteins include, but are not limited to protein kinases e.g., cyclin-dependent kinases (CDKs), and their activating associated subunits, namely cyclins (CYCs). Other cell cycle interacting proteins capable of forming a (heteromeric) complex include ORC1 and CDC6, CDC7 and DBF4, and E2F and DP.

The resulting complex of at least two cell cycle interacting proteins capable of forming a (heteromeric) complex is termed a "cell cycle complex". A preferred cell cycle complex is a CDK/cyclin complex, namely the complex formed when a preferably functional cyclin associates with its appropriate CDK,

preferably a functional form thereof. Such complexes may be active in phosphorylating proteins and may or may not contain additional protein species. Alternatively preferred cell cycle complexes are ORC1/CDC6 or CDC7/DBF4 or E2F/DP.

The invention includes modified forms of a cell cycle interacting protein, including homologues or analogues (as defined below) thereof. A preferred modified cell cycle interacting protein is a modified CDK protein wherein the modification removes the inhibitory effect of phosphates on CDC2, in particular the threonine-14 and/or tyrosine-15 have been substituted with non-phosphorylatable residues such as phenylalanine and/or alanine. Examples of such modified CDKs include Cdc2aA14F15 and Cdc2bA14F15. Another form of modification includes the mutation of the amino acid residue responsible for ATP binding, namely the D residue is replaced with an N residue to form for example Cdc2b.N161, Cdc2f.N164, or Cdc2aN147.

Another preferred modified cell cycle interacting protein is a modified cyclin protein wherein the modification results in the stablisation of the cyclin. Such modification may be the result of the mutation or complete or partial removal of the destruction box (D box) motif (RxxLxx[L/I]xN) in the cyclin N-terminal domain (where R and L residues are highly conserved and x stands for any amino acid) (Plesse et al 1998). Examples of modified cyclins for use in the present invention include CYCA2;2Δ64, CYCA2;3Δ63, CYCB2;1Δ44 (where Δ – is the truncated form lacking the N-terminal part containing the destruction box).

"Homologues" of cell cycle interacting proteins are those peptides, oligopeptides, polypeptides, proteins and enzymes which contain amino acid substitutions, deletions and/or additions relative to a non-mutant or wild-type cell cycle interacting protein polypeptide, without altering one or more of its cell cycle control properties. To produce such homologues of cell cycle

interacting protein, amino acids present in the protein can be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break helical structures or sheet structures, and so on.

Substitutional variants are those in which at least one residue in the cell cycle interacting protein amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the cell cycle interacting protein. Insertions can comprise amino- terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the cell cycle interacting protein sequence.

Amino acid variants of the cell cycle interacting polypeptide may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at

predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

"Analogues" of a cell cycle interacting protein are defined as those peptides, oligopeptides, polypeptides, proteins and enzymes which are functionally equivalent to a cell cycle interacting protein.

Analogues of a cell cycle interacting protein include those CDKs, cyclins etc and modified versions thereof that comprise peptides, polypeptides, proteins, and enzymes that are capable of functioning in a plant cell and/or plant tissue and/or plant organ and/or whole plant to produce the same modified plant growth and/or yield and/or architectural characteristics as the ectopic expression and/or (over)expression of such cell cycle interacting proteins forming a cell cycle complex.

The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occurring nucleotides with an analog.

The present invention denotes nucleic acid molecules which enhances expression of said cell cycle interacting proteins. For example, said nucleic acid molecules comprise a coding sequence of a mentioned protein or of a regulatory protein, e.g., a transcription factor, capable of inducing the expression of said cell cycle interacting protein.

The term "regulatory sequence" as used herein denotes a nucleic acid molecule increasing the expression of the said protein(s), e.g. of cell cycle interacting protein(s), due to its integration into the genome of a plant cell in close proximity to the gene(s), e.g. encoding said cell cycle interacting protein(s). Such regulatory sequences comprise promoters, enhancers, inactivated silencer intron sequences, 3'UTR and/or 5'UTR coding regions, protein and/or RNA stabilizing elements or other gene expression control elements which are known to activate gene expression and/or increase the amount of the gene products.

The introduction of said nucleic acid molecule(s) leads to de novo expression, or if the mentioned regulatory sequence(s) is used to increase in expression of said proteins, resulting in an increased amount of active protein in the cell. Thus, the present invention is aiming at providing de novo and/or increased activity of e.g., cell cycle interacting proteins.

The experiments performed underlying the current invention clearly show that overexpression of *CYCB1;1* and *CDC2a* in conjunction results in a growth stimulation of both root and shoot of between 10% to 30%. This growth stimulation requires the overexpression of both genes, since there was no growth stimulating effect of overexpression of either gene alone; and in the case of *CDC2a* overexpression alone there is a growth reduction. The relative reduction in growth rate of the roots in the CDC2a overexpressing lines increases in function of time. Growth rates 3 days after sowing were similar.

F1 seedlings overexpressing both CYCB1;1 and CDC2a exhibited increased growth. This increase was apparent 3 days after sowing (earliest measurement) throughout the entire observation period.

It was found that the growth increase is not due to more rapid germination of the seeds, since no difference in timing of germination was observed and the general growth rate pattern with a flattening of the curve at 11 days after germination was maintained.

Further, it was found that increased cell number rather than increased cell size explains the observed increase in growth (see figure 6).
Furthermore, support has been found that the increased cell number is due to a more rapid cell division rate rather than to a larger number of dividing cells in the meristem. This explanation is coherent with a role of the protein kinase and its regulatory protein in the control of cell division.

Whereas in the current invention, transgenic lines overexpressing both CDC2a and CYCB1;1 were obtained by crossing a line homozygous for a 35ScaMV-CDC2a construct with a line homozygous for a modified 35S-CYCB1;1 construct, it is clear for a person skilled in the art that the same effect could also be obtained by introducing in one plant e.g., a DNA construct in which both the CDC2a and CYCB1;1 are placed under a constitutive or tissue specific promoter.

As is evident from the above, in one embodiment of the method of the present invention said nucleic acid molecule(s) encode(s) said cell cycle interacting protein(s) and the regulatory sequence(s) is (are) capable of increasing the expression of a gene encoding said cell cycle interacting protein(s). This means, that a nucleic acid molecule comprises a coding sequence for a cell cycle interacting protein as defined herein.
A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

As has been demonstrated in the appended examples, one of said cell cyclin interacting proteins is a protein kinase. Therefore, in a preferred embodiment

of the method of the present invention one of said cell cycle interacting proteins is a protein kinase.

In a particularly preferred embodiment of the method of the present invention said protein kinase is a cyclin-dependent kinase (CDK) and the other of said cell cycle interacting proteins is a cyclin. CDKs and cyclins that can be employed according to the invention are described in Table 1, in Segers et al., 1997 or Renaudin et al., 1996, (the disclosure contents of which are hereby incorporated by reference). Preferred coexpression of combinations of CDKs and cyclins in a cell cycle complex and the resulting phenotype are described below:

1. A-type CDK with a cyclin D2 or cyclin D4: Without being bound by any theory or mode of action overexpression of A-type CDKs and cyclin D2 and/or cyclin D4 shortens the G1 phase and overrides the checkpoints, which monitors the availability of sugars. As such plants complete their lifecycle (from seed to seed) faster. It also is expected that plants can complete extra rounds of cell division resulting in an enhanced production of biomass. By using meristem specific promoters (e.g. promoters active in root- or shoot meristems, or subdomains thereof, promoters active during early seed development, cambium specific promoters etc.) growth of particular organs can be enhanced.

2. A-type CDK with a cyclin D4: Without being bound by any theory or mode of action overexpression of A-type CDKs and cyclin D4 elevates the threshold that cells needs to re-enter the cell cycle. As such plants cells are more easily regenerated and form more structures (such as lateral roots) of which the formation is dependent on the re-activation of the cell cycle (G0 to G1 transition).

3. A-type CDK with a D-type cyclin: The G1/S checkpoint as well as the G2/M checkpoint is of importance for the arrest of cell division under environmental stress conditions. It is anticipated that overexpression of A-type CDKs and D-type cyclins would result in plants with growth characteristics that are more tolerant to stress conditions which cause a cell cycle arrest at the G1/S boundary.

4. A-type CDK with a B-type cyclin or a B-type CDK with a B-type cyclin: overexpression of A-type and/or B-type CDKs with B-type cyclins overrides the G2/M checkpoints. Such plants are expected to have growth characteristics less sensitive to environmental stress conditions, such as osmotic stress and complete faster the G2 phase. Furthermore, cells will become less dependent upon the continuous availability of cytokinins.

5. A-type CDK with a D-type cyclin: Without being bound by any theory or mode of action the G1 phase is thought to be of importance for growth control of plant cells and an arrest of cells at the G1 phase (e.g. through the use of a dominant negative A-type CDK) cause cells to become larger. As such it is anticipated that co-expression of A-type CDKs and D-type cyclins enhances the progression through the G1 phase and reduces the average cell size. More cells per unit surface results in a modification of the tissue texture (relative more cell wall material, more membranes etc.) and has an important impact on quality traits. For example the difference between spring and summer wood in trees is largely a consequence of differences in cell size. Possibly fibrelength of cells (e.g. cotton fibres) can be modified through an alteration of the length of the G1 phase.

6. B-type CDK with an A-type cyclin: plant A-type cyclins are expressed from mid S till early M phase, therefore it can be expected that the co-expression of CDC2bAt with CYCA results in an enhanced progression though these cell cycle phases. Also cells may become less sensitive to the G2/M checkpoint

control, making the plants putatively less sensitive to stress conditions and plant growth regulators which operate at this transition point. The result would be that these plants grow better in suboptimal conditions. compared to normal plants, noting that suboptimal conditions may occur frequently even under good agronomical conditions. A higher yield is anticipated under most, but particularly under suboptimal/marginal, agricultural conditions.

The following Table 2 also illustrates preferred CDK/cyclin cell cycle complexes for use in the performance of the application:

TABLE 2

CYCLIN	CDK
CYCA2;1	Cdc2a
CYCA2;2	Cdc2a, Cdc2b, Cdc2f, Cdc2bN161 Cdc2aN146
CYCA2;3	Cdc2b, Cdc2f, Cdc2bN161, Cdc2aN146
CYCB1;1	Cdc2a, Cdc2b
CYCB1;2	Cdc2a, Cdc2b
CYCB2;1	Cdc2b; Cdc2f
CYCB2;2	Cdc2a, Cdc2b
CYCD1;1	G1-CDK, Cdc2a
CYCD2;1	G1-CDK, Cdc2a
CYCD3;1	G1-CDK, Cdc2a
CYCD4;1	Cdc2a; Cdc2b, Cdc2f, Cdc2bN161 Cdc2aN146, Cdc2fN164

It may not be necessary to express or induce the expression of native wild type cell cycle interacting proteins, such as above described CDKs and cyclins but it may be sufficient to provide for complex formation of at least a catalytic and/or regulatory subunit of said cell cycle interacting proteins. Therefore, another embodiment of the method of the present invention said nucleic acid molecule(s) encode(s) at least a catalytic and/or regulatory subunit of said cell cycle interacting protein(s).

Components of CDK/cyclin complexes that can be employed in accordance with the method of the present invention and how to obtain them are known to the person in skilled and are described, e.g., in WO 98/41642, WO 92/09685 the disclosure of which is hereby incorporated by reference.

One aspect of the invention provides a method of modifying plant growth and/or yield and/or architecture by expressing in particular cells, tissues or organs of a plant, at least two subunits of a cell cycle complex operably under the control of a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissues-specific promoter sequences and organ-specific promoter sequences. Examples of such promoters include promoters which are:

- **stem**-expressible and more specifically in the stem cambium: to increase strength and thickness of a plant stem to confer improved stability and wind-resistance on the plant
- **meristem** expressible: to inhibit or reduce apical dominance or increasing the bushiness of a plant. This is a desirable phenotype in a number of crop plants for example in the different Brassica species.
- **tuber** expressible: to increase or improve tuber production in the plant
- **seed** expressible: to increase seed production in plants in particular to increase seed set and/or seed production and/or seed yield.
- **endosperm** expressible: those skilled in the art will be aware that grain yield in crop plants is largely a function of the amount of starch produced in the endosperm of the seed. The amount of protein produced in the endosperm is also a contributing factor to grain yield. In contrast that embryo and aleurone layers contribute little in terms of the total weight of the mature grain. Therefore endosperm-expressible promoters provide the advantage of increasing grain size and grain yield of the plant.
- **root** expressible: to increase or enhance the production of roots or storage organs derived from roots
- **nodule** expressible: to increase the nitrogen-fixing capability of a plant.

- **embryo** expressible: embryo size being important for growth after germination
- **leaf** expressible
- **flower** expressible
- **fruit** expressible

Examples of promoters suitable for use in gene constructs of the present invention include those listed in Table 3, amongst others. The promoters listed in Table 3 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention. Table 4 describes constitutive promoters for use in the present invention.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive, glucocorticoid-responsive or dexamethasone-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible, glucocorticoid-inducible, or dexamethasone-inducible expression respectively, on said nucleic acid molecule.

In case the above-described cell cycle interacting proteins or at least one of them are to be expressed de novo, it is preferred to employ in the method of the present invention genes encoding such cell cycle interacting proteins, wherein said gene is expressible in plant cells. Thus, in another embodiment the method of the present invention said nucleic acid molecule(s) is (are) operatively linked to control sequences allowing the expression of the nucleic acid molecule(s) in the plant. Said control sequences comprise a promoter,

enhancer, silencer, intron sequences, 3'UTR and/or 5'UTR regions, protein and/or RNA stabilizing elements. Preferably, said control sequence is a chimeric, tissue specific, constitutive or inducible promoter.

Preferably, both proteins are expressed under the control of a promoter which is active in non differentiated plant cells or in plant protoplasts growing in an artificial medium. The increased growth rate of the cells results in increasing growth of the plant cells in plant cell culture, thus allowing the production of more biomass in plant cell culture. Plant cell production in plant culture can be useful for the production of certain secondary metabolites of plants which may be useful in the pharmaceutical, cosmetics, food industry etc.

The present invention further relates to a nucleic acid molecule encoding at least two cell cycle interacting proteins as mentioned above.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that comprise the nucleic acid molecule or at least two nucleic acid molecules and/or regulatory sequences according to the invention. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Plasmids and vectors to be preferably employed in accordance with the present invention include those well known in the art. Alternatively, the nucleic acid molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

In a preferred embodiment the vector of the present invention comprises separate nucleic acid molecules encoding at least one of said cell cycle interacting proteins.

In a preferred embodiment the nucleic acid molecule present in the vector is linked to (a) control sequence(s) which allow the expression of the nucleic acid molecule or of cell cycle interacting proteins in a host cell, e.g. prokaryotic and/or eukaryotic cells.

The term "control sequence" refers to regulatory DNA sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoter, ribosomal binding site, and terminators. In eukaryotes generally control sequences include promoters, terminators and, in some instances, enhancers, transactivators or transcription factors. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components. Preferably, said control sequence comprises a constitutive, chimeric, tissue specific or inducible promoter.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is used.

Thus, the vector of the invention is preferably an expression vector. An "expression vector" is a construct that can be used to transform a selected host cell and provides for expression of a coding sequence in the selected host. Expression vectors can for instance be cloning vectors, binary vectors or integrating vectors. Expression comprises transcription of the nucleic acid

molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise normally promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV). Other promoters commonly used are the polyubiquitin promoter, and the actin promoter for ubiquitous expression. The termination signals usually employed are from the Nopaline Synthase promoter or from the CAMV 35S promoter. A plant translational enhancer often used is the tobaccos mosaic virus (TMV) omega sequences, the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Mait, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676). Additional regulatory elements may include transcriptional as well as translational enhancers. Advantageously, the above-described vectors of the invention comprises a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygro, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor

Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a vector of the invention.

As it is immediately evident to the person skilled in the art, the vectors used according to a method of the present invention can carry nucleic acid molecules encoding the above-mentioned enzymes or enzymatical fragments thereof and fusions of targeting signals to these molecules. The same applies to the above described plant cells, plant tissue and plants transformed therewith. Likewise, said nucleic acid molecules may be under the control of the same regulatory elements or may be separately controlled for expression. In this respect, the person skilled in the art will readily appreciate that the nucleic acid molecules encoding e.g. the domains of cell cycle interacting protein(s) can be expressed in the form of a single mRNA as transcriptional and optionally translational fusions. This means that domains are produced as separate polypeptides or in the latter option as a fusion polypeptide that is further processed into the individual proteins, for example via a cleavage site for proteinases that has been incorporated between the amino acid sequences of both proteins. The resultant protein domains can then self-assemble in vivo. Of course, the domains may also be expressed as a bi- or multifunctional polypeptide, preferably disposed by a peptide linker which advantageously allows for sufficient flexibility of both proteins. Preferably said peptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of one of

said proteins and the N-terminal end of the other of said proteins when said polypeptide assumes a conformation suitable for biological activity of both proteins when disposed in aqueous solution in the plant cell. Examples of the above-described expression strategies can be found in the literature, e.g., for dicistronic mRNA (Reinitiation) in Hefferon (1997), Brinck-Peterson (1996) and Hotze (1995); bifunctional proteins are discussed in Lamp (1998) and Dumas (1997) and for linker peptide and protease refer to Doskeland (1996).

The present invention furthermore relates to host cells comprising a vector as described above or the mentioned complex overexpressed in a plant cell according to the invention wherein the nucleic acid molecule is foreign to the host cell.

By "foreign" it is meant that the nucleic acid molecule is either heterologous with respect to the host cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of said nucleic acid molecule. This means that, if the nucleic acid molecule is homologous with respect to the host cell, it is not located in its natural location in the genome of said host cell, in particular it is surrounded by different genes. In this case the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The vector or nucleic acid molecule according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally.

The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae.

In another embodiment of the present invention, a composition comprising vectors wherein each vector contains at least one nucleic acid molecule encoding at least one cell cycle interacting protein is disclosed. The expression of said vectors results in the production of at least two cell cycle interacting proteins and assembly of the same in a complex in vitro or in vivo.

Another object of the invention is a method for the preparation of a cell cycle complex which comprises the cultivation of host cells according to the invention which, due to the presence of a vector or a nucleic acid molecule according to the invention, are able to express such a cell cycle complex, under conditions which allow expression of the cell cycle complex and recovering of the so-produced complex from the culture.

Accordingly, the present invention relates to a cell cycle complex obtainable by said method of the present invention or encodable by the nucleic acid molecule of the present invention.

The term "expression" means the production of a protein or nucleotide sequence in the cell. It includes transcription into an RNA product, post-transcriptional modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications. The terms "protein" and "polypeptide" used in this application are interchangeable. "Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus peptides and oligopeptides are included within the definition of polypeptide. This term does also refer to or include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages,

as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

Methods for the introduction of foreign DNA into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment, pollen-mediated transformation, plant RNA virus-mediated transformation, liposome-mediated transformation, transformation using wounded or enzyme-degraded immature embryos, or wounded or enzyme-degraded embryogenic callus and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow for stably integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example co-transformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).
Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1

(pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, Agrobacterium mediated transformation etc.

Plants may also be transformed by an *in planta* method using *Agrobacterium tumefaciens* such as that described by Bechtold *et al.*, (1993) or Clough *et al* (1998), wherein *A. tumefaciens* is applied to the outside of the developing flower bud and the binary vector DNA is then introduced to the developing microspore and/or macrospore and/or the developing seed, so as to produce a transformed seed without the exogenous application of cytokinin and/or

gibberellin. Those skilled in the art will be aware that the selection of tissue for use in such a procedure may vary, however it is preferable generally to use plant material at the zygote formation stage for *in planta* transformation procedures.

The term "transformation" as used herein, refers to the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for the transfer. The polynucleotide may be transiently or stably introduced into the host cell and may be maintained non-integrated, for example, as a plasmid, or alternatively, may be integrated into the host genome. The resulting transformed plant cell or plant tissue can then be used to regenerate a transformed plant in a manner known by a skilled person.

In general, the plants which can be modified according to the invention and which either show overexpression of a cell cycle complex according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as a crop plant, root plant, oil producing plant, wood producing plant, agricultured bioticultured plant, fodder or forage legume, companion plant, or horticultured plant e.g., such a plant is wheat, barley, maize, rice, carrot, sugar beet, cichorei, cotton, sunflower, tomato, cassava, grapes, soybean, sugar cane, flax, oilseed rape, tea, canola, onion, asparagus, carrot, celery, cabbage, lentil, broccoli, cauliflower, brussel sprout, artichoke, okra, squash, kale, collard greens or potato.

Thus, the present invention also relates to transgenic plants and plant tissue comprising transgenic plant cells according to the invention. Due to the (over)expression of the protein complex of the invention, e.g., at developmental stages and/or in plant tissue in which they do not naturally occur or are present at low levels, these transgenic plants may show various growth, yield or

architectural modifications in comparison to wild-type plants. In other words, in one embodiment the present invention relates to a transgenic plant cell displaying de novo expressed cell cycle interacting protein complex or an increased amount of said complex compared to a corresponding wild type plant cell. Said transgenic plant cell comprises at least one nucleic acid molecule or regulatory sequence as defined above or obtainable by the method of the present invention. Furthermore, the present invention relates to transgenic plants and plant tissue obtainable by the method of the present invention. As mentioned above, said transgenic plants may display various idiotypic modifications, preferably display modified and/or accelerated and/or enhanced plant growth, root growth and/or yield compared to the corresponding wild type plant.

Preferred characteristics of the transgenic plants in the present invention is for example that the displays and increased cell division rate. In view of the general teaching of the present invention, it will be appreciated that the present invention contemplates the broad application of the inventive method to the modification of a range of cellular processes, including but not limited to the initiation, promotion, stimulation or enhancement of cell division, seed development, tuber formation, shoot initiation, leaf initiation, root growth, the inhibition of apical dominance etc.

Any transformed plant obtained according to the invention can be used in a conventional breeding scheme or in *in vitro* plant propagation to produce more transformed plants with the same characteristics and/or can be used to introduce the same characteristic in other varieties of the same or related species. Such plants are also part of the invention. Seeds obtained from the transformed plants genetically also contain the same characteristic and are part of the invention. As mentioned before, the present invention is in principle applicable to any plant and crop that can be transformed with any of the transformation method known to those skilled in the art and includes for

instance corn, wheat, barley, rice, oilseed crops, cotton, tree species, sugar beet, cassava, tomato, potato, numerous other vegetables or fruits.

In yet another aspect, the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which either contain transgenic plant cells over-expressing the protein complex according to the invention. Harvestable parts can be in principle any useful parts of a plant, for example, flowers, pollen, seedlings, tubers, leaves, stems, fruit, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

Furthermore, the present invention relates to the use of the above described nucleic acid molecules, regulatory sequences, and vectors for increasing cell division rates in plants, plant cells or plant tissue. Preferably, said increased cell division rates result in increased biomass, plant growth, root and/or shoot growth, increased seed setting. Preferably, said increased cell division rates result in increased plant growth, modified architecture and/or yield, e.g. of harvestable material, which is displayed for instance by (but not limited to) increased or enhanced biomass, root growth, shoot growth, seed set, seed production, grain yield, fruit size, nitrogen fixing capacity, nodule size, tuber formation, stem thickness, endosperm size, number of fruit per plant etc.

The method of the present invention provides plant cells, plant tissue and plants with novel phenotypes due to the increased or de novo formation of complexes of cell cycle interacting proteins. The plants, plant tissue and plant cells of the present invention will allow the understanding of function of cell cycle protein complexes during this cell division may also open up the way for finding compounds that interfere with formation of such complexes. Thus, the present invention provide a basis for the development of mimetic compounds that may be inhibitors or regulators of cell cycle interacting proteins or their encoding genes. It will be appreciated that the present invention also provides

cell based screening methods that allow a high-throughput-screening (HTS) of compounds that may be candidates for such inhibitors and regulators.

In a further embodiment the present invention relates to a composition comprising the nucleic acid molecule, the plant cell or the vector of the present invention or the mentioned vector comprised in the composition of the present invention or the mentioned nucleic acid molecules or regulatory sequences.

Further, in one embodiment the present invention relates to the use of the mentioned nucleic acid molecule or the mentioned regulatory sequence or the nucleic acid molecule, the vector or the plant cell of the present invention or the mentioned vectors of the composition of the present invention for the production of more biomass, of secondary metabolites or additives for plant culturing in plant cell culture.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The present invention is further illustrated by reference to the following non-limiting examples.

Unless stated otherwise in the examples, all recombinant DNA techniques are performed according to protocols as described in Sambrook et al. (1989), Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY or in Volumes 1 and 2 of Ausubel et al. (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

The invention is hereunder further explained by way of examples, including a material and method section, without being restrictive in the scope of the present invention.

Short description of the drawings:

Figure 1. Southern blots of the wild type (C24), and transgenic *CycB1;1* overexpressing *A. thaliana* lines (Cyc 28.10 and Cyc 5.9). Genomic DNA extracted from C24, Cyc 28.10 and Cyc 5.9 was digested with the indicated enzymes, separated on a 3% agarose gel and blotted. The membranes are hybridised with a probe derived from the CycB1;1 cDNA at high stringency.

Figure 2. RNA gel blot analysis. RNA was extracted from control plants (C24), homozygous Cyc 5.9 plants, homozygous Cyc 28.10 plants, and heterozygous Cyc 5.9 x CDC2aAt plants. 20 µg of RNA was separated on a 0.8% agarose gel and blotted on a nitro-cellulose membrane. Equal loading was confirmed by methylene blue staining. The blot was hybridised using an antisense CYCB1;1 probe.

Figure 3. CYCB1;1 (A) and CDC2aAt (B) western blots. Proteins were extracted from the indicated lines, and separated on a 12% SDS-PAGE gel. After immuno-blotting on a nitro-cellulose membrane the filters were probed using a CYCB1;1 (diluted 1/500) or a CDC2aAt (diluted 1/5000) specific antibody. As second antibody an anti-rabbit antibody coupled to peroxidase was used (diluted 1/10000). The detection was performed using the chemoluminescent procedure (Pierce, Rockford, IL).

Figure 4. Total root growth between day 2 and day 11. Genotypes are indicated as follows: c24, wild type; 5.9 and 28.10, independent *CYCB1;1* overexpressing lines and cdc2a, 35S-*CDC2a*; the crosses "x" indicates female x male. Data indicate mean ± SE (n = 8-13).

Figure 5. Root elongation rates as a function of time after sowing. Genotypes are indicated as in Fig 4. Data are averages ± SE (n =8 –13) from the same roots as in Fig 4.

Figure 6. Length distribution of cortical cells along the roots of wild type (C24) and F1 seedlings of the cross between *CYCB1;1* and *CDC2a* over expressing lines on day 9. Data are averages ± SE (n = 2).

Figure 7. Area of the shoot in lateral projection at day 13 as determined from the image of the shoot on the culture plates. Genotypes are indicated as in Fig 4. Data are averages ± SE (n =8 –13) from the same roots as in Fig 4.

The examples illustrate the invention without being intended as limiting:

EXAMPLES

General methodology for growth analysis in *Arabidopsis thaliana*.

Seeds of wild type *A. thaliana* (C24) and all transgenic lines are harvested from plants growing in the same conditions (22 °C and continuous light: 110 µE m^{-2} s^{-1} PAR) and stored at 4°C after harvesting from completely dried plants. For each line to be tested the following screens were performed comparing wild-type and transgenic.

Screen 1. Root growth.

At day minus 3 (d–3) seeds were surface sterilised with 15% household bleach and plated on the surface of agar-solidified modified Hoagland solution (containing 4mM KNO_3, 1mM Ca $(NO_3)_2$, 2mM KH_2PO_4, 0.3mM $MgSO_4$, 0.09µM FeEDTA, 46.26µ HBO_3, 9.91µM $MnCl_2$, 0.77µM $ZnSO_4$, 0.31µM $CuSO_4$, 0.11µM $NaMoO_3$, 0.1% (w/w) Sucrose and 0.8% (w/w) plant tissue culture agar (Lab M, Bury, England) in 12x12 cm square tissue culture plates, which were then placed vertically in the dark at 4°C for 3 days. On day 0, the plates transferred to a growth chamber with constant conditions (22 °C and 80 µE m^{-2} s^{-1} PAR).

After germination, usually at day 2-3, the position of the tip of the main root was marked daily at the bottom of the plate with a razorblade. At day 11, the plates were digitised using a flatbed scanner with overhead illumination attached to a PC. Files with a resolution of 9.775 pixels per mm were saved in Tiff format.

Daily growth of individual roots on the plates was determined by measuring the distance along the main root axis between successive marks, using the

freehand tool of the image analysis program Scion Image (Beta 3, Scion Corporation, Maryland, USA). The obtained data were transferred to the spreadsheet program Excel (Microsoft Corp). Total root growth over the observation period was obtained by adding all measured distances for each root. Root elongation rate for each root as a function of time was determined by dividing daily growth by the time interval between successive marks.

Estimation of the effect on cell division was done by measuring mature cell length. To this end the roots were whole mounted in the same nutrient solution specified above without the agar. Cortical cells were visualized with DIC optics in the region where root hairs have just reached their mature length using a microscope (Zeiss Axioscope fitted with a Zeiss Fluar 20X na = 0.75 lens). Typically, 5 average growing roots from the same experiment as used for determination of root elongation rate were selected. In each of these, the length of at least 20 mature cortical cells was measured on-line in the image analysis program Scion Image. For this, images were captured with a DCC camera (COHU 4910, USA) mounted on the microscope and connected to a PC with a framegrabber board (Scion LG3, Scion Corporation, Maryland, USA). The rate of cell production by the meristem was estimated from the ratio of the average root growth rate determined above, and mature cortical cell length.

In limited cases, cortical cell length was determined over the whole growth zone. Using the same setup as for the mature cell length, a series of partially overlapping images was recorded covering the whole of the growth zone and part of the mature region. Each series was transformed into a single composite image, which was then used to measure the length of all cells in each cortical file, starting from the quiescent centre. These data were transformed to express the length of each cell as a function of its midpoint. Interpolation and smoothing of these data was performed with a specially created algorithm, which repeatedly fits polynomials to small sections of the

data to estimate the midpoint of such section (Beemster and Baskin, 1998). Data obtained with this algorithm are equidistally spaced and were averaged between replicate roots. All data processing was done using the spreadsheet program Excel (Microsoft Corporation). This procedure gives, in addition to the mature cell length, also an idea about size of meristematic cells, and size of the meristem.

Screen 2: Total leaf area.

Total leaf (shoot) area was determined from the scanned images of the root systems described above. This was done by thresholding the image so as to select the entire shoot of each plantlet and then using the "analyse particles" routine. Obtained area values contain both leaf blade, petiole and hypocotyl. They are an underestimation for the true value of the area of these parts as the blades are aligned randomly instead of parallel to the field of view and there is overlap between various plant parts. It is obvious that the degree of underestimation increases for larger plants (more and larger organs) and therefore observed differences between genotypes are a conservative estimation of the true magnitude of differences in leaf area.

Screen 3: Leaf initiation and appearance rate.

At day 0 seeds were surface sterilised with 15% household bleach and plated on the surface of agar-solidified modified full strength Murashige and Skoog solution (Micro and macro elements (Duchefa)), 0.1% (w/w) Sucrose and 0.6% (w/w) plant tissue culture agar (Lab M, Bury, England) in 14.5 cm round / 2.5 cm high tissue culture plates (Falcon) which were placed horizontally in a growth chamber with constant conditions (22 °C and 110 μE m^{-2} s^{-1} PAR). After 7,14 and 21 days 5 average plants were selected from each line. On these plants the number of visible leaves was determined, and subsequently, by dissection under a binocular, the number of initiated leaves (= the number of visible leaves + the number of primordia).

Screen 4: Mature leaf size, cell density and estimated numb r of cells per leaf.

On the same plants from Screen 3, at day 21 all leaves were dissected and placed on a microscope slide in some water and covered with a coverslip. These slides were then scanned using the fladbed scanner (see Screen 1) and the area of each leaf was measured individually using the automated routine "analyse particles" on thresholded images in the aforementioned image analysis program Scion Image. After observation, the leaves were cleared overnight in ethanol and stored in 70% lactic acid. Cell density was determined by mounting the cleared leaves in lactic acid on a microscope slide and observing them with under DIC optics using the microscope fitted with DCC camera and connected to a PC with a framegrabber board (Scion LG3, Scion Corporation, Maryland, USA). On each leaf 4 images were captured of both adaxial epidermis and palisade parenchyma, halfway along the blade and in the middle between the mid vein and the leaf margin. On each of those images cell density was estimated by counting the number of cells in the image, whereby partial cells touching the left and upper edge were not, and those touching the right and lower edge of the image were included in the count. In the epidermis, stomata and pavement cells were counted separately. Cell density was then calculated as the number of cells divided by the area of the image. The density per blade was estimated as the average over the 4 images and the number of cells per blade of each type by multiplying this number with the total leaf area.

Screen 5: Seed weight and size.

Plants are germinated on agar as in Screen 1, transferred to soil at two weeks and all seeds are harvested when the plants have completely dried. For each plant the total seed weight is then determined. Finally seed size is determined by placing between 100 and 300 seeds per parental plant on the flatbedscanner. Images are scanned at 2400 dpi and analysed using the program Photoshop with a set of additional image analysis plug-ins (The

image processing toolkit version 3.0, Reindeer Games, Inc). The procedure is as follows: First the image is thresholded to select the seeds. Then touching seeds are separated using the watershed routine. After that all size/shape parameters are determined using the features/measure all command. From the resulting file the columns containing area, length, breadth, formfactor and roundness are selected. Outliers (dust and contamination particles) are removed based on their deviating formfactor and roundness factor. Of the remaining seeds the distribution is plotted and mean, median, average, standard deviation and standard error of the mean are determined.

EXAMPLE 1

Construction of the binary vector PGSC-TCyc1

Vector pcyc1T735 (gift of Dr. Paulo Ferreira, Departemento de Bioquímica Médica, UFRJ, Rio de Janeiro, Brazil), a PUC 19 vector (Yanish-Perron, 1985) containing 1.2Kb *CYCB1;1* cDNA with a T7 leader peptide and a NOS terminator, was digested with the restriction enzymes NcoI and XbaI. The resulting sequence consisting of 1.2Kb *CYCB1;1* cDNA, a T7 leader peptide and NOS terminator, was subsequently cloned into the vector TXGUS, (De Veylder et al 1998), from which the *GUS* gene had been excised with NcoI and XbaI, resulting in vector TXCyc1. TXCyc1 was digested with EcoRI and blunt cloned into the binary vector PGSC 1704, that carries hygromycine resistance, by means of the SnabI site, giving rise to vector PGSC-TCyc1.

EXAMPLE 2

Agrobacterium-mediated DNA transfer and plant transformation.

The PGSC TCyc1 vector was mobilised by the helper plasmid pRK 2013 into *Agrobacterium tumefasciens* C58C1RifR, harbouring the plasmid PGV 2260 (Deblaere, 1985). *A. thaliana* plants (ecotype C24) were then transformed by

root transformation (Valvekens, 1988). Transgenic plants were selected on hygromycine containing media and later transferred to soil for optimal seed production. A segregation analysis of ten independent lines was performed in the F1 generation based on hygromycine resistance, and out of two parental lines with single locus insertion (1/4 segregation; line 5 and 28) two homozygous daughter lines (5.9 and 28.10) of the F2 generation were selected. Southern blotting confirmed independency of the transformants (Fig. 1). To verify if lines 5.9 and 28.10 are actually overexpressing *CycB1;1*, a Northern (Fig. 2) and Western (Fig. 3) blots were performed using a *CYCB1;1* specific probe or antibody, respectively.

Both of these *CYCB1;1* overexpressing lines were crossed with a transgenic homozygous line of *A. thaliana*, containing the *CDC2a*At cDNA under control of a CaMV 35S promoter (Hemerly, 1995).

EXAMPLE 3

Series of reciprocal crosses

A series of reciprocal crosses (i.e. with each line functioning once as the pollen parent and once as seed parent) were made between the *CYCB1;1* overexpressing lines 5.9 and 28.10 and 35S-*CDC2a* and between these lines and the wild type. All seeds were harvested from a single batch of plants grown in a growth chamber with constant conditions (22 °C and 110 µE m^{-2} s^{-1} PAR), to avoid effects of the growth conditions of the mother plants on the progeny being analysed.

Root growth and cell length distribution was determined using Screen 1 described above. Leaf (shoot) area was determined using Screen 2 described above.

Total root growth

Overexpression of *CYCB1;1* does not result in an increased root growth between day 2 and day 11 (Fig. 4), whereas overexpression of *CDC2a* in this

experiment even resulted in a 33% growth reduction. Interestingly, the F1 of the crosses between the *CYCB1;1* and *CDC2a* overexpressing lines grew 20 – 23% faster than the wild-type, with the exception of the 35S-*CDC2a* x 28.10 cross which grew 14% slower. For the 5.9 x 35S-*CDC2a* cross these results were consistent. Although not all combinations were tested, the similarity of the growth between the wild-type and the crosses of the overexpression lines with the wild-type indicate that for growth stimulation the combined overexpression of both *CDC2a* and *CYCB1;1* is required under these conditions.

Root elongation rates

In accordance with the absence of overall growth differences over the period between day 2 and day 11 between the wild type and the two *CYCB1;1* overexpressing lines (Fig 4), no significant difference in growth rate between these lines and the wild type are observed at any time during the observation period (Fig 5a). The reduced growth of the *CDC2a* overexpressing line appears to be associated with a reduced acceleration over time rather than a proportional difference throughout the growth period (Fig 5a). In contrast, the seedlings overexpressing both *CYCB1;1* and *CDC2a*, grow proportionally faster than the wild type throughout the growth period (Fig 5b). The reduced growth in the of the cross between *CDC2a* and *CYCB1;1* overexpressing lines with the 35S-*CDC2a* as maternal line seems to be due to a reduced acceleration compared to the wild type, because the growth rates shortly after germination are very similar (Fig 5b). This pattern is similar to that observed for the *CDC2a* overexpressing line (Fig 5a).

Cell length distribution

Variations in root elongation can be due to differences in cell expansion or cell division characteristics. To get a first indication about the cellular basis of the observed growth enhancement, the length distribution of cortical cells was analysed along the root. Typically small meristematic cells are found at the tip

of the root. In both wild type and roots from F1 seedlings from the *CYCB1;1* and *CDC2a* overexpressing lines this region is approximately 500 □m long (Fig 6). Next to the meristem, a region of rapidly growing cells is located between approximately 500 and 1750 µm from the quiescent centre of the root for both genotypes. Basal to 1750 µm, cells have reached their mature cell size. Given the small sample size of 2 roots per genotype and the large standard errors for the F1 line, it is uncertain if the observed differences in cell size are real.

Shoot growth

The area of the shoot in lateral projection mirrors the differences found in root growth (cf. Figs 3 and 6). Although only a rough measure for shoot area, these data indicate that the growth increase in the *CYCB1;1* and *CDC2a* overexpressing lines is not restricted to the roots, but also occurs in the aerial parts of the plant.

EXAMPLE 4

Weight of seeds

In addition to the effect of the overexpression of *CYCB1;1* and *CDC2A* on vegetative growth the following experiment is performed to investigate its generative growth. For this, seeds from the same reciprocal crosses are sown germinated on plates with agar solidified medium as specified for Example 3. Two weeks after germination, individual seedlings are planted individually in pots with moist potting mix and placed in a growth chamber with constant conditions (22 °C and 110 µE m^{-2} s^{-1} PAR). For each plant, individual seed pods are harvested when ripe, but before it opens in order to prevent seeds from getting lost. Counts are made for each plant of the number of seeds in a number of pods in predetermined positions. The total seed weight for each plant is weighed and the average seed weight is determined by weighing 100 seeds from each plant. Seed weight and size are also determined according to the methods described in Screen 5.

EXAMPLE 5

Leaf growth under natural light conditions

In order to establish that the observed growth enhancement is not dependent on the specific growth conditions utilised for Example 3, an experiment similar to Example 3 is performed, but with a day/night cycle of 8/16 hrs. In addition to this, leaf growth under natural light conditions on soil (natural light intensity is too high for root growth) is investigated. For this, seeds of the same crosses as used in Example 3 are sown directly into potting soil and placed in glasshouse. Leaf area are measured at 1 weekly intervals from 5 representative plants of each cross. For this the leaf blades are dissected and placed on a flatbed scanner, which make a digital image of the leaves. Total leaf area is determined for each plant by measuring the combined blade area's using the thresholding option of the image analysis program Scion Image.

Similarly, to examine the growth of plants under suboptimal conditions, coexpressing transgenic plants (e.g. Cdc2b and CycA) are grown on media with 0.5X NaCl – thus providing an environmental stress or more particularly a salt stress. The response of the transgenic plants to this stress condition are assessed using the various Screens described previously.

EXAMPLE 6

Yield Increase in Rice: Production of transgenic rice plants overexpressing cdc2Os-1 and cycOs2

1. Cloning of the cDNAs encoding cdc2Os-1 and cycOs2

The nucleotide sequences encoding cdc2Os-1 and cycOs2 have been published (Sauter *et al.*, 1995). The corresponding cDNAs are cloned using the RT-PCR technique. The primer pair used to amplify the cdc2Os-1 is CCATGGAGCAGTACGAGAA (SEQ ID NO:1) for the 5' side and

CAGTGTCATTGTACCATCTCAA (SEQ ID NO:2) for the 3' side. The expected size of the amplification product is 891 bp. The template is total RNA isolated from a rice IR 52 cell suspension culture (Lee *et al.* 1989) that has been previously reversed transcribed as a bulk. The PCR conditions chosen to amplify this sequence are: 40 cycles of denaturation at 92° for 10 sec, annealing at 60° for 10 sec, extension at 72° for 60 sec.

Concerning the oligos for cycOs2, they match ATGGAGAACATGAGATCTGA (SEQ ID NO:3) for the 5'end and TTACAGTGCCACGCTCTTGAG (SEQ ID NO:4) for the 3'side of the sequence. The expected size of the amplification product is 1259 bp. The following PCR conditions are used: 45 cycles of denaturation at 92° for 10 sec, annealing at 53° for 10 sec, and extension at 72° for 90 sec.

The Pfu polymerase is used in both cases to generate blunt end fragments.

2. Subcloning of the maize ubiquitin promoter into a binary vector

The maize ubiquitin promoter from plasmid pAHC17 (Christensen, 1996) is excised as PstI fragment (made blunt ended with Pfu polymerase) and subcloned into the XbaI site (filled-in) of the binary vector pBIBHYG to give the vector pBHU.

3. Subcloning of the cdc2Os-1 and cyclinOs2 cDNAs into the pBHU binary vector

The cDNA of cdc2Os-1 and cyclinOs2 are cloned into the SacI site (trimmed off) of pPHU to produce pBHU-cdc2 and pBHU-cyc2. The vectors are then introduced into an *Agrobacterium tumefaciens* octopine strain, via electroporation (McCormac, 1998).

4. Rice transformation via *Agrobacterium*

A. tumefaciens bearing either pBHU-cdc2 or pBHU-cyc2 are used to produce transgenic rice expressing either cdc2Os-1 or cyclinOs2 under control of the ubiquitin promoter, following Hiei Y., (1994). Lines expressing highest levels

of the transgenes are crossed to produce transgenic lines co-expressing both transgenes.

5. Expected results

On the basis of results obtained in *Arabidopsis* it is anticipated that transgenic rice plants overproducing both cdc2Os-1 and cyclinOs2 will display increased growth rates and robustness. Since the growth stimulating effects observed in *Arabidopsis* have rather general character and not confined to a particular organ or tissue, we expect also the transgenic rice to show an increase in the grain size.

EXAMPLE 7

Mutant alleles and wild type genes of CDC2bAt and CDC2fAt

Mutant alleles and wild type genes of CDC2bAt and CDC2fAt were cloned under the control of the CaMV 35S promoter and transferred to a binary vector. The CDC2bAt and CDC2fAt genes are cloned in a kanamycin-containing vector. The mutant alleles include dominant negative forms of CDKs (CDC2b.N161 and CDC2fAt.N164 - in both constructs the D residue was replaced with an N residue; this mutation has been shown to inactivate the kinase causing an arrest of the cell cycle) and positive forms of the CDKs. The following cyclin genes are cloned in a hygromycin vector allowing the coexpression of the cyclins and CDK combinations by crossing.

1. Plasmid constructions

CDC2bAt constructs:

NcoI and BamHI restriction sites were introduced in the cDNAs of CDC2bAt, CDC2bAt.A14F15 and CDC2bAt.N161 by performing PCRs with the following primers: 5'-GGCCATGGAGAAGTACGAGAAGC-3' (SEQ ID NO:5) (containing a NcoI restriction site) and 5'-GGGGATCCTCAGAACTGAGACTTGTCAAGG-3' (SEQ ID NO:6) (containing a BamHI restriction site). The different PCR fragments were cut with NcoI and

BamHI and cloned into the NcoI and BamHI sites of pH35S (Hemerly et al., 1995). The cassettes 35S-CDC2bAt-3'NOS, 35S-CDC2bAt.A14F15-3'NOS and 35S-CDC2bAt.N161-3'NOS were cloned into the EcoRI and SalI restriction sites of the binary vector pBinPlus (Engelen, 1995 Transgenic Research, 4:288-290)

CDC2fAt constructs:

ClaI and SalI restriction sites were introduced in the cDNAs of CDC2fAt, CDC2fAt.A26F27 and CDC2fAt.N164 by performing PCRs with the following primers: 5'-GGATCGATATGGACGAGGGAGTTATAGC-3' (SEQ ID NO.7) (containing a ClaI restriction site) and 5'-GGGGGAAGCACAGTCGACATATGC-3' (SEQ ID NO:8) (containing a SalI restriction site. The different PCR fragments were cut with ClaI and SalI and cloned into the ClaI and SalI sites of the binary vector pGV1990.

CYCA2;2 constructs:

in order to introduce the ClaI and SalI restriction sites and a HA tag into the cDNAs of CYCA2;2 and CYCA2;2Δ64, PCRs were performed using the following primers:

5'-GCGATCGATATGGGCTACCCTTACGATGTTCCAGATTACGCTATGTA TTGCTCTTCTTCGATGC-3' (SEQ ID NO:9) (containing a ClaI site and a HA tag in fusion with the full length *CYCA2;2*),

5'-GCGATCGATATGGGCTACCCTTACGATGTTCCAGATTACGCTACCTCT GCAGATATTATTTATTC-3' (SEQ ID NO:10) (containing a ClaI site and a HA tag in fusion with the truncated *CYCA2;2Δ64*) and

5'-GGCGTCGACGTTGCTTGGTGTCATCTTG-3' (SEQ ID NO: 11) (containing a SalI restriction site).

The different PCR fragments obtained were cut with ClaI and SalI and cloned into the ClaI and SalI sites of pGV1990.

CYCA2;3 constructs:
in order to introduce the SalI restriction sites and a HA tag into the cDNAs of *CYCA2;3* and *CYCA2;3Δ63*, PCRs were performed using the following primers:

5'-
GCGGTCGACATGGGCTACCCTTACGATGTTCCAGATTACGCTATGGGG
AAGGAAAATGCTG-3' (SEQ ID NO:12) (containing a SalI site and a HA tag in fusion with the full length *CYCA2;3*),

5'-
GCGGTCGACATGGGCTACCCTTACGATGTTCCAGATTACGCTGTTAACT
CCAATACAGC-3' (SEQ ID NO:13) (containing a SalI site and a HA tag in fusion with the truncated *CYCA2;3Δ63*) and

5'- GGCGTCGACGGTTAGGAGTTGAAACC-3' (SEQ ID NO:14) (containing a SalI restriction site).

The 3'NOS was removed from pH35S by BamHI and XbaI digestion and cloned into the BamHI and XbaI restriction sites of the vector pLBR19, resulting into the pLBR19/NOS vector. The obtained PCR fragments were cut with SalI and cloned into the SalI restriction site of pLBR19/NOS. By restriction with the KpnI site, the cassettes 35S-CYCA2;3-3'NOS and 35S-CYCA2;3Δ63-3'NOS were removed from pLBR19/NOS, blunt ended and cloned into the SNABI restriction site of the pGSC1704 binary vector.

CYCB2;1 constructs:

in order to introduce the SalI and BamHI restriction sites and a HA tag into the cDNAs of *CYCB2;1* and *CYCB2;1Δ44*, PCRs were be performed using the following primers:

5'-
GCGGTCGACATGGGCTACCCTTACGATGTTCCAGATTACGCTATGGTTA
ACTCATGCGAG-3' (SEQ ID NO: 15) (containing a SalI site and a HA tag in fusion with the full length *CYCB2;1*),

5'-
GCGGTCGACATGGGCTACCCTTACGATGTTCCAGATTACGCTCAGAATC
TCGCTGGTGC-3' (SEQ ID NO:16) (containing a SalI site and a HA tag in fusion with the truncated *CYCB2;1Δ44*) and

5'- CCGGATCCTGTACAAACTACTTAC-3' (SEQ ID NO: 17) (containing a BamHI restriction site).

The obtained PCR fragments were cut with SalI and BamHI and cloned into the SalI and BamHI restriction sites of pLBR19/NOS. By restriction with the KpnI site, the cassettes 35S-CYCB2;1-3'NOS and 35S-CYCB2;1Δ44-3'NOS were removed from pLBR19/NOS, blunt ended and cloned into the SNABI restriction site of the pGSC1704 binary vector.

CYCB-typeΔ construct:
in order to introduce the NcoI and BamHI restriction sites and a HA tag into the cDNA of *CYCB-typeΔ*, a PCR was performed using the following primers:

5'-
GCGCCATGGGCTACCCTTACGATGTTCCAGATTACGCTCCACATATCCG
TGATGAGG-3' (SEQ ID NO:18) (containing a NcoI site and a HA tag in fusion with the truncated *CYCB-typeΔ*), and

5'- GCGGATCCATTCTTCTCCCATTTTGG-3' (SEQ ID NO:19) (containing a BamHI restriction site).

The PCR fragment was cut with NcoI and BamHI and cloned into the NcoI and BamHI sites of pH35S. The cassette 35S-*CYCB-typeΔ*-3'NOS were removed from pH35S by EcoRI and BamHI digestion, blunt ended and cloned into the SNABI restriction site of the pGSC1704 binary vector.

2. Plant transformation

The different constructs were transferred into *Agrobacterium tumefaciens* and introduced into *Arabidopsis thaliana* ecotype C24 by the floral dip method (Clough, 1998).

3. Analysis

Transgenic plants are analysis for various growth and cell division characteristics or phenotypes according to the Screens described in the General Methodology above.

EXAMPLE 8

Evidence of cell cycle protein complexes

1. Purification protocol of native CDK complexes

All steps are carried out at 4°C or on ice; at all stages the presence of CDK complexes is followed based on their kinase activity or via western analysis.

Cell suspension cells and buffers:

five fold diluted homogenization buffer: the concentration of all additives is five times lower than in homogenization buffer except for Tris and NaCl which are the same (respectively 25 and 85 mM)

DEAE buffer: 50mM Tris.HCl (pH= 7.8 or 9.3) plus 5mM $MgCl_2$, 5mM EGTA, 5mM β-glycerophosphate, 1mM NaF, 0.01% NP40, 1mM DDT,

0.25mM PMSF, 1μg/ml aprotonin and leupeptin, 0.1mM benzamidine and $NaVO_4$.

S200 buffer: 50mM Tris.HCl (pH= 7.8 or 9.3) plus 15mM $MgCl_2$, 5mM EGTA, 5mM β-glycerophosphate, 1mM NaF, 1mM DDT, 0.1mM $NaVO_4$, 100mM NaCl.

Total extract: in homogenization buffer (85mM NaCl), typically 70ml

Cleansing step: DEAE Sepharose FF (Pharmacia): flow through collection into 150ml superloop (including washing of the column in five fold diluted homogenization buffer) flow 10ml /min.

Affinity binding onto Cks homologues coupled to an affinity matrix:

The columns are connected in series in this specific order: P9 (Ckshs1) 10ml, P13 (Suc1) 3ml and P10 (Cks1At) 10ml. The sample (150ml) is loaded at a flow rate of 200μl/min.

The columns (still connected in series) are then washed with bead buffer to minimize non-specific interactions (cf. higher salt concentration) at a flow rate of 500μl/min.

In this way all CDC2a binds to the P9 column. CDC2b binds to both P13 and P10.

CDK- complex elution via excess of free ligand:

Affinity columns are disconnected from one another and eluted individually with an excess of their respective free ligand (applied in DEAE buffer, pH=7.8).

Preparation of an excess of free ligand is done as follows: lyophilized powder is dissolved in 6M urea in 25mM Tris pH=7.5 as to obtain a highly concentrated solution (typically 25mg/ml); then desalted on a Sephadex G25 column (1x10cm) to DEAE buffer (PH=7.8) and slowly injected onto the affinity columns in reversed flow mode at a flow rate of 200μl/min, thus displacing the bound proteins complexes which are collected.

2. Separation of affinity eluted CDK-complexes:

The CDK containing fractions are first concentrated on DEAE 650S (TSK) therefore the pH is raised to 9.3 and the sample is applied onto the column (HR5/5 Pharmacia). Bound proteins are eluted in one step through injection of 0.5M NaCL in DEAE buffer (pH 7.8).

Then the concentrated CDK fraction is further separated by size on a gel filtration column: Superdex 200pg Pharmacia (1.7x100cm column Omnifit) or a Sephacryl S200 Pharmacia (1.5x100cm column Pharmacia). The columns are equilibrated in S200 buffer when fractions (5ml) are collected or in DEAE buffer when the eluting proteins are immediately eluted onto DEAE 650S (TSK) (HR5/5 columns), in the latter case the pH of the size exclusion buffer was raised to 9.3 (cf. concentration step).

Separately collected CDK containing fractions (5ml) which are later bound on DEAE or those CDK complexes which are directly bound to DEAE when eluting from the size exclusion column are eluted in a similar way: a 10 column-volume gradient of 0–500mM NaCl is applied and the eluting complexes are collected.

A final purification step consists of hydrophobic interaction chromatography: The conductivity of the purified fractions is raised to100mS by adding saturated ammonium sulphate and the samples are individually applied onto Ether PW-5 or Phenyl PW-5 (TSK). The bound complexes are eluted with a decreasing $(NH_4)_2SO_4$ gradient and can be tested on their kinase activity.

3. Protein complexes purified

In the CDC2a fraction (eluted from CKShs1) there were two 100kDa combinations: CDC2a with CycB2;2 and with CycA2;2. Higher molecular weight complexes containing CDC2a were also detected.

In the CDC2b fraction obtained from CKS1At both CycB1;1 and cyclin A2;2 were detected in 100kDa complexes, suggesting their association with CDC2b. CycB1;1 was also detected in a 200kDa complex and a higher molecular weight complex.

Some complexes are not bound during the affinity purification.
Cyclin A2:1 is present in a 100kDa complex with an as yet unidentified protein(s). However it is not yet clear whether the latter one is a CDC homologue or an (un)related protein.

REFERENCES

Bechtold, N.J., *et al., C.R. Acad. Sci. (Paris, Sciences de la vie/ Life Sciences) 316:* 1194-1199, 1993.

Beemster GTS, Baskin TI (1998) Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. Plant Physiol 116: 515-526

Burssens et al (1998) The cell cycle in *Arabidopsis*. Plant Physiol. Biochem. 36: 9-19.

Bögre, L., Zwerger, K., Meskiene, I., Binarova, P., Csizmadia, V., Planck, C., Wagner, E., Hirt, H., and Heberle-Bors, E. (1997) The cdc2Ms kinase is differentially regulated in the cytoplasm and in the nucleus. Plant Physiol. 113:841-852.

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5(3):213-218

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J (England), 16(6) p735-43

Dahl, M., Meskiene, I., Bögre, L., Cam Ha, D.T., Swoboda, I., Hubmann, R., Hirt, H., and Heberle-Bors, E. (1995) The D-type alfalfa cyclin gene cycMs4 complements G1 cyclin–deficient yeast and is induced in the G1 phase of the cell cycle. Plant Cell 7:1847-1857

Deblaere R, Bytebier B, De Greve H, Debouk F, Shell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid derived vectors for *Agrobacterium* mediated gene transfer to plants. *Nucleic Acid Research. Res.* 13: 4777-4788

De Veylder, L., Segers, G., Glab, N., Van Montagu, M., and Inzé, D. (1997a) Identification of proteins interacting with the Arabidopsis Cdc2aAt protein. J. Exp. Bot. 48:2113-2114.

De Veylder L; de Almeida Engler J; Burssens S; Manevski A; Lescure B; Van Montagu M; Engler G; Inze D (1999) A new D-type cyclin of Arabidopsis thaliana expressed during lateral root primordia formation. Planta 208(4):453-62.

Doerner P, Jorgensen J-E, You R, Steppuhn J, Lamb C (1996) Control of root growth and development by cyclin expression. Nature 380: 520-523

Ferreira PCG, Hemerly AS, de Almeida Engler J, Van Montagu M, Engler G, Inzé D (1994) Developmental expression of the Arabidopsis cyclin gene *cyc1At*. Plant Cell 6: 1763-1774

Foard DE, Haber AH (1961) Anatomic studies of gamma radiated wheat growing without cell division. Amer J Bot 48(6): 438-446

Francis, D., Dudits, D., and Inzé, D. (1998). Plant Cell Division. (London: Portland Press).

Hemerly AS, Ferreira P, de Almeida Engler J, Van Monagu M, Engler G, Inzé D (1993) *Cdc2a* expression in *Arabidopsis* is linked with competence for cell division. Plant Cell 5: 1711-1723

Hemerly AS, de Almeida Engler J, Bergounioux C, Van Montagu M, Engler G, Inzé D, Ferreira P (1995) Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. EMBO J 14: 3925-3936

Hiei Y., Ohta S., Komari T., Kumashiro T. (1994). Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. 6:271-282

Ivanov VB (1994) Root growth responses to chemicals. Sov Sci Rev D Physicochem Biol 13: 1-70

Labbe JC *et al*, *EMBO J. 8*:3053-3058, 1989.

Leatherwood J (1998) Emerging mechanisms of eukaryotic DNA replication initiation. Current Opinion in Cell Biology, 10:742-748

Lee L, Schzoll RE, Grimes HD, Hodges TK (1989) Plant regeneration from indica rice (Oryza sativa L.) protoplasts. Planta 178: 325-333

Liang C, Weinreich M. Stillman B (1995) ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. Cell 81(5) p667-76

Magyar, Z., Bakó, L., Bögre, L., Dedeolu, D., Kapros, T., and Dudits, D. (1993) Active cdc2 genes and cell cycle phase–specific cdc2-related kinase complexes in hormone-stimulated alfalfa cells. Plant J. 4:151-161.

Magyar, Z., Mészáros, T., Miskolczi, P., Deák, M., Fehér, A., Brown, S., Kondorosi, E., Athanasiadis, A., Pongor, S., Bilgin, M., Bakó, L., Koncz, C., and Dudits, D. (1997). Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronized alfalfa cells. Plant Cell 9, 223-235.

McCormac AC, Elliott MC, Chen DF (1998) A simple method for the production of highly competent cells of Agrobacterium for transformation via electroporation. Mol Biotechnol 9(2):155-159

Mews, M., Sek, F.J., Moore, R., Volkmann, D., Gunning, B.E.S., and John, P.C.L. (1997). Mitotic cyclin distribution during maize cell division: Implications for the sequence diversity and function of cyclins in plants. Protoplasma 200, 128-145.

Murray AW, Kirschner MW (1989) Dominoes and clocks: the union of two views of the cell cycle. Science, 246(4930) p614-21

Murray A, Hunt T (1993) The cell cycle. An introduction. Vol W.H. Freeman and Company, New York, pp 251

Plesse, B., Fleck, J., and Genschik, P. (1998). The ubiquitin-dependent proteolytic pathway and cell cycle control. In Plant Cell Division, D. Francis, D. Dudits, and D. Inzé, eds (Colchester, UK: Portland), pp. 145–163.

Renaudin, J.-P., Doonan, J.H.- Freeman, D., Hashimoto, J., Hirt, H., Inzé, D., Jacobs, T., Kouchi, H., Rouzé, P., Sauter, M., Savouré, A., Sorrell, D.A., Sundaresan, V., and Murray, J.A.H. (1996). Plant cyclins: A unified nomenclature for plant A-, B- and D-type cyclins based on sequence organisation. Plant Mol. Biol. 32, 1003-1018.

Riou-Khamlichi C; Huntley R; Jacqmard A; Murray JA (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science 5:283(5407):1541-4

Sauter M, Mekhedov SL, Kende H (1995) Gibberellin promotes histone H1 kinase activity and the expression of cdc2 and cyclin genes during the induction of rapid growth in deepwater rice internodes. Plant J 7(4): 623-632

Segers G, Gadisseur I, Bergounioux C, de Almeida Engler J, Jacqmard A, Van Montagu M, Inzé D (1996) The *Arabidopsis* cyclin-dependent kinase *cdc2bAt* is preferentially expressed during S and G2 phases of the cell cycle. Plant J. 10, 601-612

Segers G, Rouzé P, Van Montagu M, Inzé D (1997) In: Plant cell proliferation and its regulation in growth and development. Bryant JA, Chiatante D, editors. Chichester: John Wiley & Sons: p.1-19.

Shaul, O., Mironov, V., Burssens, S., Van Montagu, M., and Inzé, D. (1996). Two *Arabidopsis* cyclin promoters mediate distinctive transcriptional oscillation in synchronized tobacco BY-2 cells. Proc. Natl. Acad. Sci. USA 93, 4868-4872.

Soni, R., Carmichael, J.P., Shah, Z.H., and Murray, J.A.H. (1995) A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell 7:85-103

Sorrell, D.A., Combettes, B., Chaubet-Gigot, N., Gigot, C., and Murray, J.A.H. (1999) Distinct cyclin D genes show mitotic accumulation or constant levels of transcripts in tobacco Bright Yellow-2 cells. Plant Physiol. 119:343-351

Swenson *et al*, *Cell 47:*861-870, 1986.

Yanish-Perron C, Viera J, Messing J (1985). *Gene* 33, 103-119

Valvekens D, Van Montagu M, Van Lijsebettens M (1988) Agrobacterium tumefasciens-mediated transformation of Arabidopsis thaliana root

explants by using kanamycine selection. Proc. Natl. Acad. Sci. USA 85: 5536-5540

TABLE 1 – Examples of cyclins in plants

Class of cyclin	Typical phase dependence[a]	Examples[b]	Original name	Comments	Reference
A1	S/G2/M	Zeama;CYCA1;1 Nicta;CYCA1;1	cycIIZm ntcyc25	Zeama;CYCA1;1 triggers frog oocyte maturation; Nicta;CYCA1;1 rescues G1 cyclin deficiency in budding	see Renaudin et al (1996) Plant Mol. Biol. 32: 1003-1018
A2	S/G2/M	Nicta;CYCA2;1 Medsa;CYCA2;1[c]	ntcyc27 cycMs3	Medsa;CYCA2;1 expression suppresses the α-pheromone-induced cell cycle arrest in yeast. Medsa;CYCA2;1 and Nicta;CYCA2;1 complement G1 cyclin deficiency in yeast	see Renaudin et al (1996) Plant Mol. Biol. 32: 1003-1018; Day and Reddy (1998) Plant Mol. Biol 36: 451-461
A3	S/early G2	Catro;CYCA3;1	CYS	Catro;CYCA3;1 rescues G1 cyclin deficiency in yeast	see Renaudin et al (1996) Plant Mol. Biol. 32: 1003-1018
B1	G2/M	Arath;CYCB1;1 Arath;CYCB1;2 Catro;CYCB1;1 Nicta;CYCB1;2 Zeama;CYCB1;1 Zeama;CYCB1;2 Glyma;CYCB1;1	cyc1At cyc1bAt CYM NycycZ9 cyclaZm cyclbZm S13-6	Arath;CYCB1;1, Zeama;CYCB1;1, Zeama;CYCB1;2, and Glyma;CYCB1,2 trigger frog oocyte maturation. Arath;CYCB1;2, Catro;CYCB1,1, and Nicta;CYCB1;2 rescue G1 cyclin deficiency in yeast	see Renaudin et al (1996) Plant Mol. Biol. 32: 1003-1018
B2	G2/M	Arath;CYCB2;2 Zeama;CYCB2;1 Medsa;CYCB2;2	cyc2bAt cycIIIZm cycMs2	Zeama;CYCB2;1 triggers oocyte maturation; Medsa;CYCB2;2-immunoprecipi	Hemerly et al (1994) PNAS 91:11313-11317; Renaudin et al (1994) PNAS 91: 7375-7379;

		Orysa; CYCB2;1 Orysa;CYCB2;2		tated kinase activity is maximal in G2	Meskiene et al (1995) Plant Cell 7: 759-771;
D1	Unknown	Arath;CYCD1;1 Antma; CYCD1;1 Heltu;CYCD1;1	cyclin δ1	Rescues G1 deficiency in yeast; associates with CDC2aAt in the two-hybrid system	Soni et al (1995) Plant Cell 7:1847-1857; Antma and Heltu - unpublished data see: Sorrell et al (1999) Plant Physiol. 119: 343-351
D2	Non specific	Arath;CYCD2;1 Nicta;CYCD2;1 Cheru;CYCD2;1	cyclin δ2	Rescues G1 deficiency in yeast Expression sucrose inducible; Nicta;CYCD2;1 transcript peaks during M	Sorrell et al (1999) Plant Physiol. 119: 343-351; Soni et al (1995) Plant Cell 7:1847-1857; Renz et al (1997) Plant Physiol. 113: 1004.
D3	Non specific	Arath;CYCD3;1 Medsa;CYCD3;1 Nicta;CYCD3;1 Nicta;CYCD3;2 Antma;CYCD3;2 Antma;CYCD3;1 Heltu;CYCD3;1	cyclinδ3 cycMs4	Rescues G1 deficiency in yeast. Expression cytokinin inducible; Nicta;CYCD3;1 transcript peaks during M. Expressed in only a subset of proliferating cells; interacts with Rb and ICK1	Riou-Khamlichi et al (1999) Science 283: 1541-1544 (Antma, A. majus; Heltu, Helianthus tuberosus – unpublished data see: Sorrell et al (1999) Plant Physiol. 119: 343-351
D4	Unknown	Arath;CYCD4;1		Expression sucrose inducible; expressed during lateral root primordia formation	De Veylder et al 1999, Plant, 208: 453-462

a At the transcriptional level.
b Nomenclature according to Renaudin et al. (1996).
c Expressed in a non-specific manner.

TABLE 3

EXEMPLARY TISSUE SPECIFIC FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
α-amylase (*Amy32b*)	aleurone	Lanahan, M.B., *e t al., Plant Cell 4:*203-211, 1992; Skriver, K., *et al. Proc. Natl. Acad. Sci. (USA) 88:* 7266-7270, 1991
cathepsin β-like gene	aleurone	Cejudo, F.J., *et al. Plant Molecular Biology 20:*849-856, 1992.
Agrobacterium rhizogenes rolB	cambium	Nilsson *et al., Physiol. Plant. 100:*456-462, 1997
PRP genes	cell wall	http://salus.medium.edu/mmg/tierney/html
AtPRP4	flowers	http://salus.medium.edu/mmg/tierney/html
chalene synthase (chsA)	flowers	Van der Meer, *et al., Plant Mol. Biol. 15*, 95-109, 1990.
LAT52	anther	Twell et al Mol. Gen Genet. 217:240-245 (1989)
apetala-3	flowers	
chitinase	fruit (berries, grapes, etc)	Thomas *et al.* CSIRO Plant Industry, Urrbrae, South Australia, Australia; http://winetitles.com.au/gwrdc/csh95-1.html
rbcs-3A	green tissue (eg leaf)	Lam, E. *et al., The Plant Cell 2:* 857-866, 1990.; Tucker *et al., Plant Physiol. 113:* 1303-1308, 1992.
leaf-specific genes	leaf	Baszczynski, *et al., Nucl. Acid Res. 16:* 4732, 1988.
AtPRP4	leaf	http://salus.medium.edu/mmg/tierney/html
Pinus cab-6	leaf	Yamamoto *et al., Plant Cell Physiol. 35*:773-778, 1994.
SAM22	senescent leaf	Crowell, *et al., Plant Mol. Biol. 18:* 459-466, 1992.
R. japonicum nif gene	nodule	United States Patent No. 4, 803, 165
B. japonicum nifH gene	nodule	United States Patent No. 5, 008, 194
GmENOD40	nodule	Yang, *et al., The Plant J. 3:* 573-585.
PEP carboxylase (PEPC)	nodule	Pathirana, *et al., Plant Mol. Biol. 20:* 437-450, 1992.
leghaemoglobin (Lb)	nodule	Gordon, *et al., J. Exp. Bot. 44:*

		1453-1465, 1993.
Tungro bacilliform virus gene	phloem	Bhattacharyya-Pakrasi, *et al, The Plant J. 4:* 71-79, 1992.
sucrose-binding protein gene	plasma membrane	Grimes, *et al., The Plant Cell 4:*1561-1574, 1992.
pollen-specific genes	pollen; microspore	Albani, *et al., Plant Mol. Biol. 15:* 605, 1990; Albani, *et al., Plant Mol. Biol. 16:* 501, 1991)
Zm13	pollen	Guerrero et al Mol. Gen. Genet. 224:161-168 (1993)
apg gene	microspore	Twell et al Sex. Plant Reprod. 6:217-224 (1993)
maize pollen-specific gene	pollen	Hamilton, et al., Plant Mol. Biol. 18: 211-218, 1992.
sunflower pollen-expressed gene	pollen	Baltz, et al., The Plant J. 2: 713-721, 1992.
B. napus pollen-specific gene	pollen;anther; tapetum	Arnoldo, et al., J. Cell. Biochem., Abstract No. Y101, 204, 1992.
root-expressible genes	roots	Tingey, et al., EMBO J. 6: 1, 1987.
tobacco auxin-inducible gene	root tip	Van der Zaal, et al., Plant Mol. Biol. 16, 983, 1991.
β-tubulin	root	Oppenheimer, *et al., Gene 63:* 87, 1988.
tobacco root-specific genes	root	Conkling, et al., Plant Physiol. 93: 1203, 1990.
B. napus G1-3b gene	root	United States Patent No. 5, 401, 836
SbPRP1	roots	Suzuki et al., Plant Mol. Biol. 21: 109-119, 1993.
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
AtPRP4	leaves; flowers; lateral root primordia	http://salus.medium.edu/mmg/tierney/html
seed-specific genes	seed	Simon, *et al., Plant Mol. Biol. 5:* 191, 1985; Scofield, *et al., J. Biol. Chem. 262:* 12202, 1987.; Baszczynski, *et al., Plant Mol. Biol. 14:* 633, 1990.
Brazil Nut albumin	seed	Pearson, et al., Plant Mol. Biol. 18: 235-245, 1992.
legumin	seed	Ellis, et al., Plant Mol. Biol. 10: 203-214, 1988.
glutelin (rice)	seed	Takaiwa, et al., Mol. Gen. Genet. 208: 15-22, 1986; Takaiwa, et al., FEBS Letts. 221: 43-47, 1987.
zein	seed	Matzke et al Plant Mol Biol, 14(3):323-32 1990

napA	seed	Stalberg, *et al, Planta 199:* 515-519, 1996.
wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2, 1989
wheat SPA	seed	Albani *et al,* Plant Cell, 9: 171-184, 1997
wheat α, β, γ-gliadins	endosperm	EMBO 3:1409-15, 1984
barley *ltr1* promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena *et al,* The Plant Journal, 116(1): 53-62, 1998
blz2	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa *et al., Plant J. 13:* 629-640, 1998.
rice prolamin NRP33	endosperm	Wu *et al,* Plant Cell Physiology 39(8) 885-889, 1998
rice α-globulin Glb-1	endosperm	Wu *et al,* Plant Cell Physiology 39(8) 885-889, 1998
rice OSH1	embryo	Sato *et al,* Proc. Natl. Acad. Sci. USA, 93: 8117-8122, 1996
rice α-globulin REB/OHP-1	endosperm	Nakase *et al.* Plant Mol. Biol. 33: 513-522, 1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorgum α-kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	embryo	Postma-Haarsma *et al,* Plant Mol. Biol. 39:257-71, 1999
rice oleosin	embryo and aleuron	Wu *et at,* J. Biochem., 123:386, 1998
sunflower oleosin	seed (embryo and dry seed)	Cummins, *et al.*, Plant Mol. Biol. 19: 873-876, 1992
LEAFY	shoot meristem	Weigel *et al., Cell 69*:843-859, 1992.
Arabidopsis thaliana knat1	shoot meristem	Accession number AJ131822
Malus domestica kn1	shoot meristem	Accession number Z71981
CLAVATA1	shoot meristem	Accession number AF049870
stigma-specific genes	stigma	Nasrallah, et al., Proc. Natl. Acad. Sci. USA 85: 5551, 1988; Trick, *et al., Plant Mol. Biol. 15:* 203, 1990.
class I patatin gene	tuber	Liu et al., Plant Mol. Biol. 153:386-395, 1991.
PCNA rice	meristem	Kosugi *et al, Nucleic Acids Research 19:*1571-1576, 1991; Kosugi S. and Ohashi Y, *Plant Cell 9*:1607-1619, 1997.
Pea TubA1 tubulin	Dividing cells	Stotz and Long, *Plant Mol.Biol.* 41, 601-614. 1999
Arabidopsis cdc2a	cycling cells	Chung and Parish, FEBS Lett,

		3;362(2):215-9, 1995
Arabidopsis Rop1A	Anthers; mature pollen + pollen tubes	Li et al. 1998 *Plant Physiol* 118, 407-417.
Arabidopsis AtDMC1	Meiosis-associated	Klimyuk and Jones 1997 *Plant J.* 11, 1-14.
Pea PS-IAA4/5 and PS-IAA6	Auxin-inducible	Wong et al. 1996 *Plant J.* 9, 587-599.
Pea farnesyltransferase	Meristematic tissues; phloem near growing tissues; light- and sugar-repressed	Zhou et al. 1997 *Plant J.* 12, 921-930
Tobacco (N. sylvestris) cyclin B1;1	Dividing cells / meristematic tissue	Trehin et al. 1997 *Plant Mol.Biol.* 35, 667-672.
Catharanthus roseus Mitotic cyclins CYS (A-type) and CYM (B-type)	Dividing cells / meristematic tissue	Ito et al. 1997 *Plant J.* 11, 983-992
Arabidopsis cyc1At (=cyc B1;1) and cyc3aAt (A-type)	Dividing cells / meristematic tissue	Shaul et al. 1996 *Proc.Natl.Acad.Sci.U.S.A* 93, 4868-4872.
Arabidopsis tef1 promoter box	Dividing cells / meristematic tissue	Regad et al. 1995 *Mol.Gen.Genet.* 248, 703-711.
Catharanthus roseus cyc07	Dividing cells / meristematic tissue	Ito et al. 1994 *Plant Mol.Biol.* 24, 863-878.

TABLE 4

EXEMPLARY CONSTITUTIVE PROMOTERS FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
Actin	constitutive	McElroy *et al,* Plant Cell, 2: 163-171, 1990
CAMV 35S	constitutive	Odell *et al,* Nature, 313: 810-812, 1985
CaMV 19S	constitutive	Nilsson *et al., Physiol. Plant. 100*:456-462, 1997
GOS2	constitutive	de Pater *et al,* Plant J Nov;2(6):837-44, 1992
ubiquitin	constitutive	Christensen *et al,* Plant Mol. Biol. 18: 675-689, 1992
rice cyclophilin	constitutive	Buchholz *et al,* Plant Mol Biol. 25(5): 837-43, 1994
maize H3 histone	constitutive	Lepetit *et al,* Mol. Gen. Genet. 231:276-285, 1992
actin 2	constitutive	An *et al,* Plant J. 10(1); 107-121, 1996